



## ORIGINAL ARTICLE

# Serological Correlate of Protection in Guinea Pigs for a Recombinant Protective Antigen Anthrax Vaccine Produced from *Bacillus brevis*

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**Abstract**

**Objective:** Recombinant protective antigen (rPA) is the active pharmaceutical ingredient of a second generation anthrax vaccine undergoing clinical trials both in Korea and the USA. By using the rPA produced from *Bacillus brevis* pNU212 expression system, correlations of serological immune response to anthrax protection efficacy were analyzed in a guinea pig model.

**Methods:** Serological responses of rPA anthrax vaccine were investigated in guinea pigs that were given single or two injections (interval of 4 weeks) of various amounts of rPA combined with aluminum hydroxide adjuvant. Guinea pigs were subsequently challenged by the intramuscular injection with 30 half-lethal doses (30LD<sub>50</sub>) of virulent *Bacillus anthracis* spores. Serum antibody titers were determined by anti-PA IgG ELISA and the ability of antibodies to neutralize the cytotoxicity of lethal toxin on J774A.1 cell was measured through the toxin neutralizing antibody (TNA) assay.

**Results:** To examine correlations between survival rate and antibody titers, correlation between neutralizing antibody titers and the extent of protection was determined. Toxin neutralization titers of at least 1176 were sufficient to confer protection against a dose of 30LD<sub>50</sub> of virulent anthrax spores of the H9401 strain. Such consistency in the correlation was not observed from those antibody titers determined by ELISA.

**Conclusion:** Neutralizing-antibody titers can be used as a surrogate marker.

## 1. Introduction

Gram-positive *Bacillus anthracis* forms highly stable spores even after excessive UV, heat, or toxic chemical introductions that affect its survival. Exposure to these

spores by cutaneous, gastrointestinal, or aerosol routes results in lethal anthrax infection [1]. Poly-D-glutamic acid capsule and anthrax toxin are the two factors that are related to the toxicity of *B. anthracis* in living organism [2]. The expression of poly-D-glutamic acid capsule,

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coded on pX02 plasmid, is controlled by *acpA* gene and the capsule itself is in charge of protecting bacteria from the immune system, especially from phagocytosis [2,3]. There are three different protein types of anthrax toxin; protective antigen (PA), lethal factor (LF), and edema factor (EF) [4]. These proteins are coded on pX01 plasmid as *pag*, *lef*, and *cya* genes respectively, and their expression is under the control of *atxA* gene [3]. Among these anthrax toxins, 83 kDa of PA cleaves into 63 kDa as it attaches to the surface of a host cell, such as a macrophage, and becomes a hydrophobic heptamer [4]. LF or EF binds on this hydrophobic surface to enter the host cell for expressing own toxicity [5]. When the concentration of EF, which is a kind of calmodulin-dependent adenylate cyclase, increases, it raises the concentration of cyclic adenosine monophosphate [6], and, therefore, chloride ions and water molecules flow out of the cell body finally causing edema of the tissue [7]. On the contrary, LF reduces the mitogen-activated protein kinase signal transduction and strengthens the level of cytokinesis tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  in macrophage cells [8]. It also elevates the amount of oxygen radicals [7] in the host cell resulting in macrophage targeted cytotoxicity, which can cause the actual cell and tissue destruction, and, ultimately, death of the organism.

Interfering with the binding of PA to LF or EF provides effective protection from anthrax infection in immunized animals [9]. Under this concept, the current trend in developing anthrax vaccine is mainly focused on PA rather than LF or EF. For example, the anthrax vaccines approved in the USA and UK are adsorbed form of purified PA obtained from the culture supernatant of nontoxigenic *B. anthracis* [9]. Anthrax Vaccine Adsorbed-Biothrax (AVA-Biothrax, formerly identified as AVA and MDPH-PA; BioPort Corporation, Lansing, MI, USA) is a widely-used anthrax vaccine in the USA which is in a form of aluminum hydroxide-adsorbed highly purified recombinant PA (rPA). However, the need for developing new vaccine is still exist as AVA-Biothrax has some drawbacks such as partial side-effects in some species and inconvenience of multiple-vaccination to achieve a decent level of immunization [10].

In this study, adsorbed form of rPA to adjuvant was tested in a guinea-pig model and their protection rate was determined to set up a reliable surrogate marker of anthrax vaccination. After single or double immunization in 4-week intervals, serum samples from immunized animals were analyzed and compared to the survival rate after virulent *B. anthracis* H9401 spore challenge.

## 2. Materials and Methods

### 2.1. Recombinant PA (rPA) preparation

rPA is purified from culture supernatant of *Bacillus brevis* 47-5Q, an asporogenic, avirulent, and non-toxigenic strain, which contains a recombinant plasmid

encoding the PA component of the anthrax toxin [11]. The host *B. brevis* 47-5Q was cultured from modified PY medium containing proteose peptone (Becton Dickinson, Sparks, MD, USA) 1%, yeast extract (Becton Dickinson) 0.5%, uracil 0.01%, glucose 1%, and 10 mg/ml erythromycin (EM). For the production of protein, it was grown at 30°C in PY broth (containing proteose peptone 1%, yeast extract 0.5%, uracil 0.01%, glucose 1%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.01%,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01%,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.001%,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.0001%). Unless otherwise specified, chemicals were obtained from Sigma (St Louis, MO, USA). Biostat-D DCU2 model (72 L, B. Braun Biotech International, Germany) was used as a fermentor for large scale bacteria culture in high concentration. Bacterial supernatant was filtered by absolute filter and flowed through anion exchange and hydrophobic interaction chromatography columns. Obtained protein was tested by SDS-PAGE, western-blot analysis, and anti-PA specific-ELISA.

### 2.2. Animals

Female Hartley guinea pigs (300 g to 320 g) were obtained from Damul Science, Jungeub City, Korea. The animal procedures were approved by the Institutional Animal Care and Use Committee of Korea National Institute of Health.

### 2.3. Vaccinations and challenge of guinea pigs

Hartley guinea pigs were inoculated intramuscularly (i.m.) with various concentrations of rPA vaccine preparation in 0.5 mL volumes as a single injection (0 week) or as two injections (0 and 4 weeks). rPA was adsorbed to Alhydrogel (2%  $\text{Al}_2\text{O}_3$ ; Brenntag Biosector, Frederikssund, Denmark) 250  $\mu\text{g}$  of aluminum per injection final concentration, for >1 hour at 4°C before use. Blood was collected for serum isolation 3 weeks. Guinea pigs were challenged in Week 4 after a single or two injections of vaccine by the i.m. route with a targeted dose of 30 half-lethal doses (30LD<sub>50</sub>) spore from *B. anthracis* H9401. The LD<sub>50</sub> of H9401 spores in Hartley guinea pigs is  $50 \pm 2$  spores [12]. The survival rates were recorded for 14 days after challenge. Spores used for the challenge were prepared according to Ivins et al [13]. Spores used in the experiment were grown in Leighton and Doi medium [14], purified by centrifugation through 58% Hypaque-76 (Amersham Health, Amersham, Buckinghamshire, UK) and suspended in 0.1% gelatin-PBS and stored at 2°C to 8°C until used.

### 2.4. Serological assay

#### 2.4.1. Anti-PA IgG ELISA

Serum samples from individual guinea pigs were analyzed for PA-specific antibody responses using an enzyme-linked immunosorbent assay (ELISA). Pre-immune serum obtained 2 days before vaccination was used as a negative control. The wells of high-binding microplates (Costar; Corning Inc., Corning, NY, USA)

were coated with 1 µg/mL of PA in 0.1 M carbonate buffer (pH 9.5) and incubated for overnight at 4°C. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBST) and blocked with 3% (v/v) bovine serum albumin in PBST. Serum samples were applied to the first column of each row and serially diluted with two-fold volumes of PBST until the last column of the row and incubated for 1 h at 37°C. PA-specific antibodies were detected using a polyclonal goat anti-guinea pig IgG conjugated to peroxidase (Sigma). After washing with PBST, color was developed using o-phenylenediamine substrate. The reaction was stopped by adding 2N H<sub>2</sub>SO<sub>4</sub>, and the absorbance at 492 nm was measured using a Sunrise microplate reader (TECAN Instruments, Grödig, Austria). The endpoint titers were defined as the reciprocal of the highest standard serum dilution that resulted in an absorbance three standard deviations greater than the average absorbance of negative control serum samples at the same dilution.

#### 2.4.2. TNA

The titer of toxin-neutralizing antibody in serum was determined using modifications to the methods of Pitt et al [15] and Little et al [4] by testing the ability of the serum to inhibit the cytotoxicity of the combination of rPA with LF (List Biological Laboratories Inc., Campbell, CA, USA). The assay was carried by exposing J774A.1 murine macrophages (ATCC TIB-67) to PA and LF in the presence or absence of serum. In brief, J774A.1 cells were plated out on 96 well plates (SPL CO. Ltd, Pyeongtaek-si, Korea) and seeded with 10<sup>6</sup> cells per well in 150 µl volumes 2 hours before testing. Cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, and 25 units of penicillin G and 25 µg of streptomycin/ml (DMEM complete). Standards and serial two-fold dilutions of samples were pre-incubated with lethal toxin (LeTx 500 ng of rPA per ml and 100 ng of LF/ml, final concentrations) in a humidified incubator at 37°C, 5% CO<sub>2</sub>, for 1 hour. Dilutions of serum sample and LeTx were prepared in DMEM complete containing 10 mM HEPES. Medium was removed from wells containing the J774A.1 cells and replaced with 100 µl per well of the sample or standard mixed with LeTx. After a 4-hour incubation at 37°C in 5% CO<sub>2</sub>, 25 µl of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide (MTT, Amresco, Solon, OH, USA) was added to each well at a final concentration of 5 mg/ml. After an additional 1-hour incubation at 37°C, the cells were lysed by adding 100 µl extraction buffer (90% isopropanol containing 25 mM HCl and 0.5% [w/v] SDS). Absorbance was then measured at 570 nm with an ELISA reader (Tecan). Each dilution of sample and standard was tested in duplicate. Six wells contained only medium and served as medium control. Six LeTx wells contained only LeTx and served as blanks. The

percent neutralization (% control) for each dilution of sample and standard was determined by calculating % control = (sample average – lethal toxin average) / (medium control average – lethal toxin average) × 100. The percent control values were plotted against each respective test dilution using a four-parameter logistic equation algorithm and neutralizing antibody titers were expressed as the reciprocal of the dilution of antiserum that neutralized the cytotoxic activity of lethal toxin on J774A.1 cells at 50% of control value (ED<sub>50</sub>) using SoftMax Pro 5.3 (Molecular Device, Sunnyvale, CA, USA).

#### 2.5. Statistical analysis

The correlation between of protection and TNA titer or anti-PA IgG titers were acquitted with linear regression analysis was employed. Acceptance criteria include (1) the  $R^2$  for the standard curve had to be  $\geq 0.95$ ; (2) the standard curve had to contain at least contiguous standards; and (3) the coefficient of variation (%CV) for the duplicate absorbance readings for standards and samples had to be  $\leq 20\%$ .

### 3. Results

#### 3.1. Serological correlate in guinea pigs after a single injection of rPA vaccine

To determine the efficacy of single vaccination, guinea pigs were given a single injection with different amount of rPA adsorbed in Alhydrogel (250 µg in 0.5 mL PBS). Four weeks after immunization, they were challenged with 30LD<sub>50</sub> of *B anthracis* H9401 spores (Table 1, Figure 1). Their serum samples were collected at 4 weeks after immunization, and were analyzed by anti-PA IgG ELISA and TNA assays to determine their serological titers. However, reasonable levels of antibody titers were not obtained in either assay, hence any significant relationship between antibody titers and the actual animal survival rate was not found. These results also support the clinical study performed by Campbell et al [16], which demonstrated that a single injection could not induce any immunological responses in human volunteers. Taken together with these experimental and clinical evidences, boosting immunization is necessary for anthrax vaccination to obtain effective protection.

#### 3.2. Serological correlate in guinea pigs after two injections of rPA vaccine

To define the serological correlate in guinea pigs with protection, animals were given different concentrations of serially diluted rPA vaccines with 250 µg of alhydrogel in 0.5 mL PBS and after 4 weeks they received another shot of vaccine. Serum samples derived from these animals at 2 week after last vaccination were analysed by anti-PA IgG titer and TNA assays and their serological titers were compared with the survival rate (Table 2, Figure 2). In

**Table 1.** Survival against an i.m. challenge and antibody response of guinea pigs after single injection of rPA vaccine

PA( $\mu$ g) dose	Survival percentage (alive/total)	Geometric mean ELISA titer (standard error)	Geometric mean ED <sub>50</sub> TNA assay titer (standard error) <sup>a</sup>
50	50 (3/6)	634.96 (1.89)	59.01 (1.27)
25	16.7 (1/6)	400.00 (1.55)	27.41 (1.31)
12.5	0 (0/6)	224.49 (1.45)	18.66 (1.82)
6.3	0 (0/6)	1.0 (1.00)	1.0 (1.00)
3.1	0 (0/6)	1.0 (1.00)	1.0 (1.00)
1.6	0 (0/6)	1.0 (1.00)	1.0 (1.00)
0 (control)	0 (0/6)	1.0 (1.00)	1.0 (1.00)

<sup>a</sup>ED<sub>50</sub>, reciprocal of the dilution that neutralized 50% of in vitro *LeTx* cytotoxicity.

Guinea pigs received single i.m. injections (0.5 ml) of rPA vaccine containing various concentrations of rPA. Four weeks later, guinea pigs were challenged by the i.m. route with a targeted dose of 30LD<sub>50</sub> spores of the *B anthracis* H9401 strain. Sera were tested in a anti-PA IgG Titer and TNA assay.

both assays, titers showed significant ( $p < 0.0001$ ) increase after the second injection and the overall titers were much higher compared to those from single immunization. And increasing tendency of serological titers with higher concentration of rPA and the survival rate was observed (Tables 1 and 2). Furthermore, 100% of animals survived with 50  $\mu$ g of rPA per dose in 4-week double immunizations, which did not happen with any of the experimental group with single immunization. These

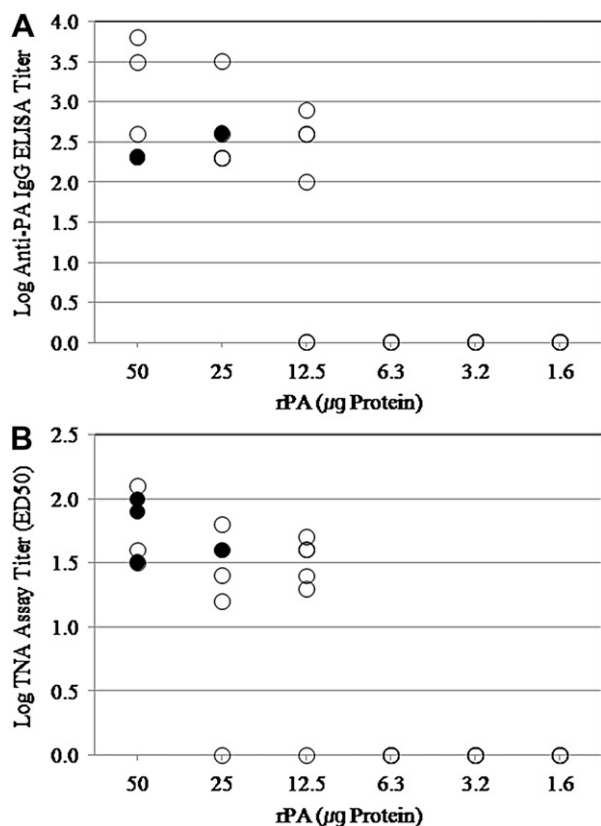
results clearly support the classical immunological concept that the boosting brings higher protection rate to challenge [17].

### 3.3. Serological titer from double vaccination produces a reliable coefficient to survival rates

To obtain correlate between serological titers and protection, serological titers of serum samples from individual animals were compared to the survival data and linear regression analysis was performed to obtain  $R^2$  values. Among two serological titers, anti-PA IgG titers from the animals of 4 week double immunization schedule did not show any correlation to the survival rate under this experimental condition ( $R^2 = 0.18$ , data not shown). On the contrary, a high correlation coefficient was obtained from TNA titers with serum samples of double immunizations ( $R^2 = 0.96$ ) (Figure 3). From the linear regression analysis, TNA titers for 50% survival against 30LD<sub>50</sub> spore challenge was 551 and for 100% survival was 1176. The correlation coefficient ensured that double immunization schedule with a 4-week interval might be able to give a good indication about vaccine efficacy. This also partially supports the previous finding that asserted admitting TNA as a surrogate marker for testing vaccine efficacy [18].

## 4. Discussion

The correlation between neutralizing-antibody titers and protection against *B anthracis* infection in different animal species vaccinated with rPA provides surrogate markers to evaluate the protective efficiency of rPA vaccines in humans [19–27]. Because of the cost associated with conducting such studies with a large enough number of nonhuman primates to achieve statistically significant results, alternative animal models have been examined. McBride et al [26] were unable to identify a correlate of protection in guinea pigs that had received three inoculations of rPA (250, 2.5, 0.25, or 0.025  $\mu$ g/dose) formulated with Alhydrogel adjuvant at



**Figure 1.** Relationship between survivals of guinea pig with a single injection of rPA vaccine after i.m. challenge with *B anthracis* H9401 spore and Week 3. (A) Anti-PA IgG titer, (B) ED<sub>50</sub> TNA assay. Guinea pigs (●) surviving or (○) dying after i.m. injection.

**Table 2.** Survival against an i.m. challenge and antibody response of guinea pigs after two injections of rPA vaccine

PA ( $\mu$ g) dose	Survival percentage (alive/total)	Geometric mean ELISA titer (standard error)	Geometric mean ED <sub>50</sub> TNA assay titer (standard error) <sup>a</sup>
50	100 (9/9)	43890.9 (1.12)	4063.8 (1.26)
25	100 (9/9)	25600.0 (1.22)	3225.4 (1.26)
12.5	88.9 (8/9)	16126.9 (1.29)	1097.3 (1.35)
6.3	80.0 (8/10)	5198.4 (1.26)	685.9 (1.18)
3.1	33.3 (3/9)	696.4 (1.72)	278.6 (1.38)
1.6	30.0 (3/10)	459.5 (1.09)	113.1 (1.37)
0 (Control)	0.0 (0/10)	1.0 (1.00)	1.0 (1.00)

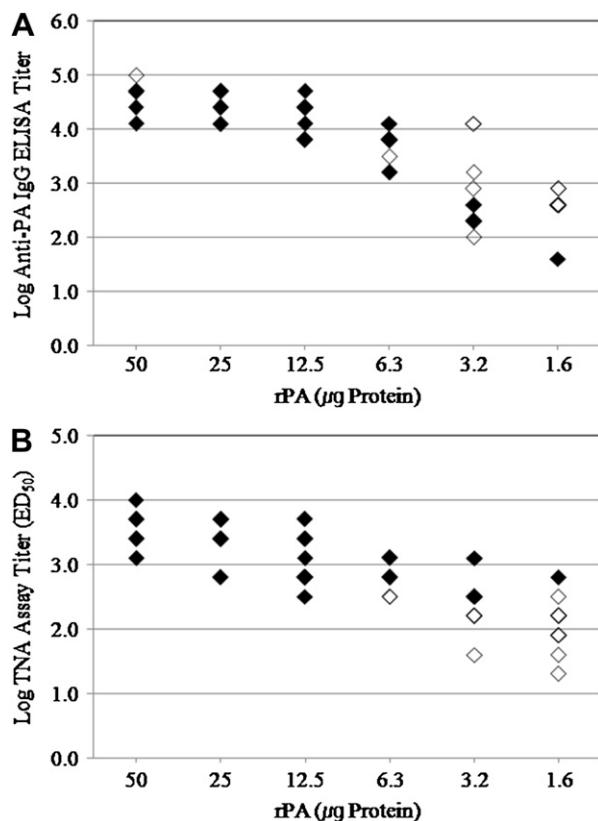
<sup>a</sup>ED<sub>50</sub>, reciprocal of the dilution that neutralized 50% of in vitro LeTx cytotoxicity.

Guinea pigs received two i.m. injections at 0 and 4 weeks (0.5 ml each) of rPA vaccine containing various concentrations of rPA. Four weeks after the last injection (Week 8), guinea pigs were challenged by the i.m. route with a targeted dose of 30LD<sub>50</sub> spores of the *B. anthracis* H9401 strain. Sera were tested in a anti-PA IgG Titer and TNA assay.

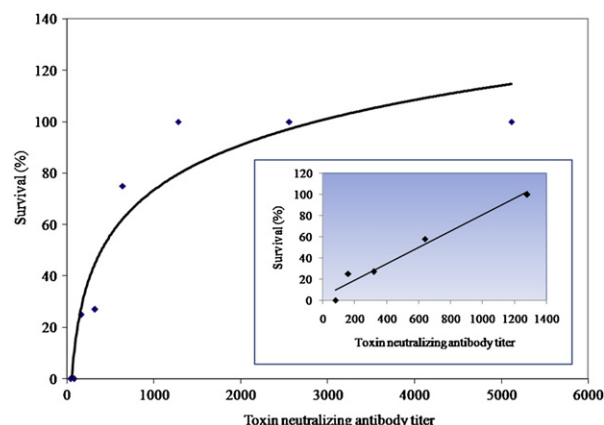
0, 2, and 4 weeks and challenged at 6 weeks by the aerosol route with the Ames strain of *B. anthracis*. Pitt et al [27] reported that survival of guinea pigs that had been challenged 3 months after being inoculated with two injection of rPA (50, 5, or 0.5  $\mu$ g/dose) formulated with alhydrogel at 0 and 4 weeks was not dose dependent. Reuveny et al [18] argued against multiple

inoculations and favored a single inoculation of vaccine. Their group demonstrated that their neutralizing antibody titers could serve as an effective correlate of protection for guinea pigs that were challenged intradermally with spores of the Vollum strain spore of *B. anthracis* after a single subcutaneous inoculation of rPA (25, 12.5, 6.3, 3.1, 1.6, or 0.8  $\mu$ g) adsorbed to Alhydrogel. The anti-PA IgG titers, however, proved to be of limited value in serving as a surrogate marker [18].

Our present study was designed to find correlations between protection level and either anti-PA IgG IgG titers or neutralizing-antibody titers. To eliminate



**Figure 2.** Relationship between survivals of guinea pig inoculated with two injection of rPA vaccine after i.m. challenge with *B. anthracis* H9401 spore and Week 7. (A) Anti-PA IgG titer; (B) ED<sub>50</sub> TNA assay. Guinea pigs (◆) surviving or (◇) dying after i.m. injection.



**Figure 3.** Correlation between TNA titers and protection after two injections. Relationship between serological titer and protection of guinea pigs with a double vaccination serially diluted of rPA vaccine at a 4-week interval. After 4 weeks after the second vaccination, guinea pigs were challenged i.m with *B. anthracis* H9401 spores. Each point in the group represents the average of duplicate determinations performed with pooled sera derived from 9 or 10 guinea pigs. TNA titers of serum samples from individual animals in each group were compared to the survival data of the animals (inset). Linear regression was performed using data from experimental cohorts with neutralizing-antibody titers of 1176 or less. The calculated  $R^2$  value is 0.96.



potential effects of other *B anthracis* derived components, we have used highly purified rPA adsorbed to alhydrogel as a vaccine. For the first step of establishing a surrogate marker for anthrax vaccine, suitable immunization intervals (4 week) were determined (data not shown). Then we tested the number of vaccinations to obtain adequate level of protection efficacy for testing rPA-alhydrogel vaccine. In the case of the single injection experiment, we obtained just 50% protection with the highest rPA dose and antibody titers were not high enough to guarantee the reproduction of this result, especially in TNA (titers < 100). Because of these reasons, single injection was not the optimal condition to examine vaccine efficacy.

With the information that a single injection is not sufficient to establish serological marker, we performed another set of experiments using two injections with a 4-week interval. In this case, it would obviously obtain higher serological titers in both assays compared to those of single injection schedule. Overall, animals that received two injections could survive more than animals with single injection and serological titers rapidly increased after the second injection. These results clearly support the classical concept that boosting brings higher serological titer and a higher protection rate to immunological challenge [17]. We could obtain relatively high correlation coefficients from anti-PA IgG and neutralizing-antibody titers with serum samples from 4 weeks injection schedule ( $R^2 = 0.18$  in anti-PA IgG ELISA,  $R^2 = 0.96$  in TNA). These correlation coefficients ensured that a two-injection schedule with a 4 week interval might be able to serum as a good surrogates about vaccine efficacy.

Our findings support the notion that antibodies involved in neutralization are also involved in protection against the full course of infection. This is in agreement with previous observations in which monoclonal antibodies capable of neutralizing the cytotoxic effect were shown to delay death in guinea pigs [28]. The results indicate that neutralizing-antibody titers can be used as a reliable correlate for protection in guinea pigs. A correlation between protection and neutralization titers was recently observed in a rabbit model [15], suggesting that this phenomenon is not species specific. Application of such markers to human studies therefore appears to be feasible, even though similar experiments in nonhuman primates would be required to gain more confidence.

In conclusion, we have produced a series of experimental data to establish a surrogate marker that can indicate rPA vaccine efficacy. According to our results, the best correlation between serological titers and animal survival rates was obtained with double immunization using a 4-week interval schedule. Under this condition, antibody titers obtained by TNA were high enough to be reproduced and  $R^2$  values higher than 0.95, which indicates that TNA can be a reliable experimental technique for estimating animal survival. Therefore, to test the

efficacy of an anthrax PA vaccine, two injections in combination of appropriate adjuvant at a 4-week interval and TNA for serological assay are recommended.

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